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Notch-1 regulates transcription of the epidermal growth factor receptor through p53

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The Notch pathway plays a key role in the development and is increasingly recognized for its importance in cancer. We demonstrated previously the overexpression of Notch-1 and its ligands in gliomas and showed that their knockdown inhibits glioma cell proliferation and survival. To elucidate the mechanisms downstream of Notch-1 in glioma cells, we performed microarray profiling of glioma cells transfected with Notch-1 small interfering RNA. Notable among downregulated transcripts was the epidermal growth factor receptor (EGFR), known to be overexpressed or amplified in gliomas and prominent in other cancers as well. Further studies confirmed that Notch-1 inhibition decreased EGFR messenger RNA (mRNA) and EGFR protein in glioma and other cell lines. Transfection with Notch-1 increased EGFR expression. Additionally, we found a significant correlation in levels of EGFR and Notch-1 mRNA in primary high-grade human gliomas. Subsequent experiments showed that p53, an activator of the EGFR promoter, is regulated by Notch-1. Experiments with p53-positive and -null cell lines confirmed that p53 partially mediates the effects of Notch-1 on EGFR expression. These results show for the first time that Notch-1 upregulates EGFR expression and also demonstrate Notch-1 regulation of p53 in gliomas. These observations have significant implications for understanding the mechanisms of Notch in cancer and development.

Introduction

The Notch pathway plays a central role in stem cell maintenance, cell fate decisions and cell survival. Four members of the Notch family have been identified, each a single-pass transmembrane protein with complex extracellular and intracellular domains. Binding of a Delta-like or Jagged ligand on one cell to Notch on an adjacent cell triggers enzymatic cleavages, which liberate the Notch intracellular domain (NICD). The NICD travels to the nucleus, where it activates transcription by a CSL transcription factor (CBF1 in human cells). This drives transcription of mediators of Notch signaling such as members of the HES and HEY transcription factor families. A non-classical pathway involving Notch and possibly mediated by the Deltex ubiquitin ligases (1) has also been suggested. While some downstream mediators of Notch signaling have been identified, it is clear that many direct and indirect Notch targets have yet to be revealed.

Abbreviations: EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; NICD, Notch intracellular domain; PCR, polymerase chain reaction; siRNA, small interfering RNA.

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In recent years, roles for Notch and its ligands have been shown in several cancers. For instance, Notch can act in an oncogenic fashion, with acute T-cell lymphoblastic leukemia and lymphoma perhaps the clearest example (2–4). In other neoplasms, such as non-small cell lung cancer and skin cancer, Notch has a tumor suppressor function (5,6). More recently, Notch has been shown by our laboratory and others to play a tumor-promoting role in brain tumors. Notch-2 has been suggested to drive embryonal brain tumor growth (7), whereas Notch-3 has been implicated in choroid plexus tumors (8). We demonstrated previously the presence of Notch-1 and its ligands Delta-like-1 and Jagged-1 in gliomas and their importance in glioma cell survival (9). To probe for downstream effectors of Notch-1 in gliomas, we performed microarray analysis of glioma cell lines following efficient small interfering RNA (siRNA) knock down of Notch-1.

Microarray data from Notch-1 knockdown revealed decreased expression of the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase implicated in carcinogenesis and in gliomagenesis. EGFR has been found to be amplified and/or upregulated in a large subset of the most aggressive gliomas (10), and EGFR inhibitors have shown efficacy in the treatment of patients with high-grade gliomas (11). In central nervous system development, EGFR and Notch have both been shown to direct cells toward a glial cell fate; however, neither has previously been shown to upregulate expression of the other. Herein, we have demonstrated the regulation of EGFR by Notch-1.

Materials and methods

Cell culture and primary human astrocytomas

Human glioma cell lines U87MG and T98G were acquired from American Type Culture Collection (Manassas, VA) and cultured in modified Eagle's medium (Mediatech, Herndon, VA,) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The 0206 cell line is a primary human glioma cell line derived from a human glioma surgical sample using an institutional review board-approved protocol and cultured in serum-free conditions using methods we have described previously (12). Human colon cancer cell lines wild-type HCT116 and p53-null HCT116 (13) were kindly provided by Dr Bert Vogelstein and cultured in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The adult human dermal fibroblast cell line was obtained from Sciencell (San Diego, CA) and grown in their media. All cell lines were incubated at 37°C in an atmosphere of 5% CO_2. Messenger RNA (mRNA) was obtained from 31 primary human gliomas as described previously (9).

Real-time polymerase chain reaction

mRNA expression levels were measured by real-time polymerase chain reaction (PCR) in a two-step procedure (reverse transcription being a separate step from the PCR) using ABI AmpliTaq Polymerase and fluorescent resonant energy transfer technology on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Reverse transcription–PCR

Cell lysis and RNA purification were done with the RNEasy kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. The reverse transcriptase reaction was done with the Superscript II kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions and PCR done using standard techniques.

Western blotting

Whole-cell lysates were prepared in Cell Signaling Lysis Buffer (Cell Signaling Technology, Danvers, MA) as per the manufacturer's instructions along with 1% sodium dodecyl sulfate and a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Indianapolis, IN). Cell lysate samples were run on a precast 4–12% Bis–Tris gel (Invitrogen), transferred to a polyvinylidene difluoride membrane (Invitrogen) and probed with primary antibody. Primary antibodies included those to EGFR antibody (1:200, Santa Cruz Biotechnology sc-03, Santa Cruz, CA), p53 antibody (1:1000, Cell Signaling Technology), glyceraldehyde 3-phosphate dehydrogenase (GAPDH)–horseradish peroxidase (1:200, Santa Cruz Biotechnology sc-20357), β-actin (1:200, Santa Cruz Biotechnology sc-1615), α-tubulin (1:5000, Sigma–Aldrich Co., St Louis, MO), p21 (1:200, Santa Cruz Biotechnology sc-6246), p53R2 (1:200, Santa Cruz Biotechnology

sc-10840) and proliferating cell nuclear antigen (PCNA) (1:200, Santa Cruz Biotechnology sc-25280). Horseradish peroxidase-conjugated secondary antibodies to rabbit, mouse and goat IgG were used (1:10 000, Jackson Immunology Laboratories, Bar Harbor, ME). All antibodies were diluted in blocking buffer (5% wt/vol non-fat dry milk or bovine serum albumin, 10 mol/l Tris–HCl, 100 mmol/l NaCl and 0.1% vol/vol Tween 20). All other buffers used during western blotting were made following the Invitrogen's NuPage protocol.

Cell transfection

Control and Notch-1 siRNA duplexes were synthesized by Xeragon (Qiagen, Valencia, CA,) p53 siRNA was obtained from Cell Signaling Technology (proprietary sequence). Cells were plated in six-well tissue culture plates at a density of 4.5×10^4 per well and transfected 24 h later with Oligofectamine (Invitrogen) according to the manufacturer's protocol, with a concentration of 10 nmol/l siRNA. A second transfection was done 24 h after the first. siRNA dosage was lowered to 10 nmol/l from the recommended concentration of 200 nmol/l because cell proliferation assays showed non-specific toxicity from control siRNA at 200 nmol/l. For the 0206 primary glioma cell line, cells were plated in laminin- and polyornithine-coated six-well plates the day prior to transfection. siRNA sequences were as follows: Notch-1—r(UGGCGGGAAGUGUGAAGCG)d(TT), r(CGCUUCACACUUCCCGCCA)d(TT); other Notch-1 siRNA (see Figure 1D)—r(CAUCCAGGACAACAUGGGC)d(TT), r(GCCC

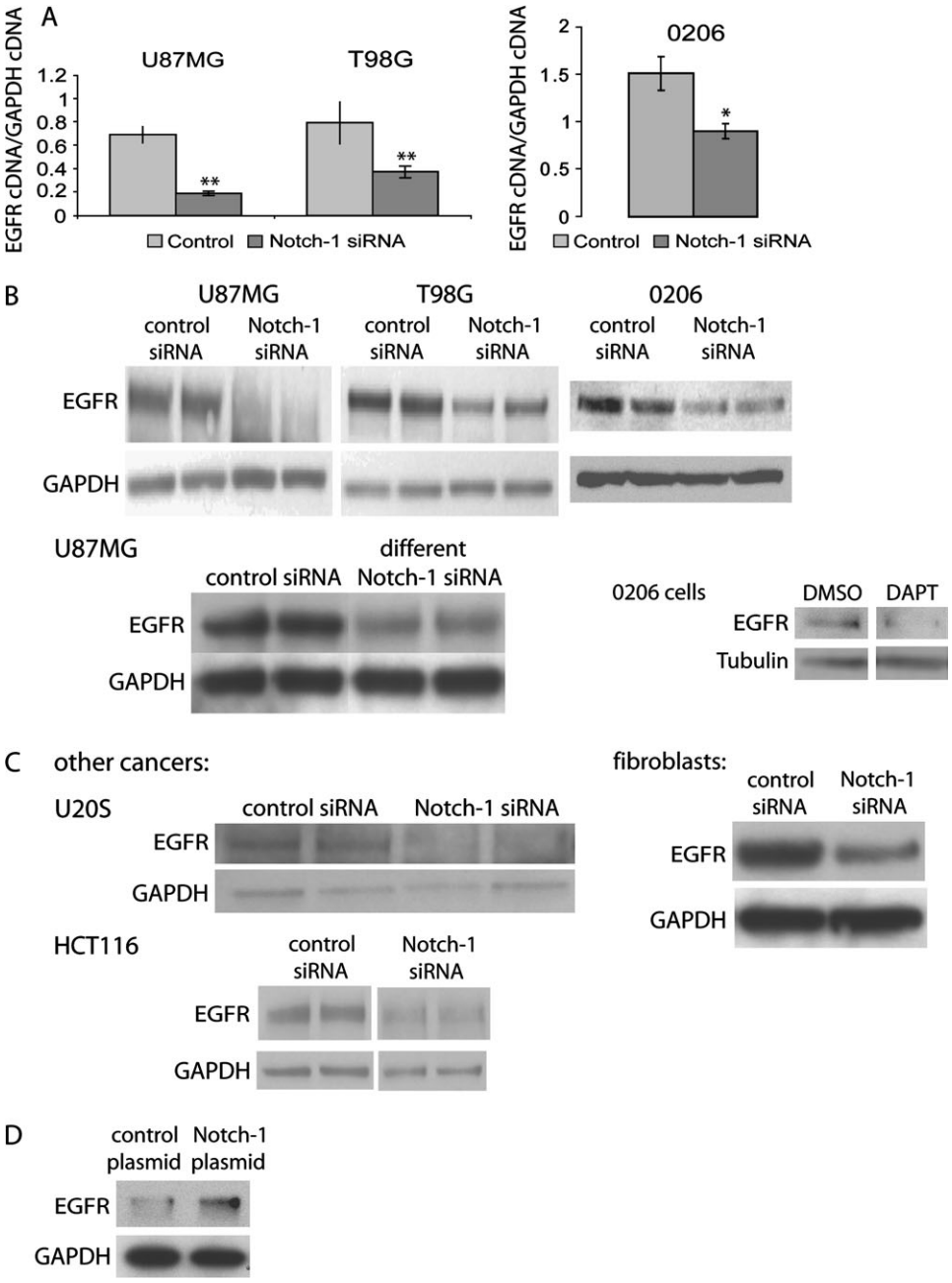

Fig. 1. Regulation of EGFR expression by Notch-1. (**A**) Real-time PCR of EGFR mRNA from two established glioma cell lines and the 0206 cell line (a primary human glioma cell line cultured in serum-free, growth factor-supplemented conditions) 72 h after first of two daily transfections with control or Notch-1 siRNA. $^{*}P$ value <0.05 and $^{**}P$ value <0.01. (**B**) Immunoblots for EGFR (and GAPDH loading control) from two established human glioma cell lines and 0206 cells 72 h after first of two daily transfections with control or Notch-1 siRNA. Immunoblots for EGFR (and GAPDH loading control) 72 h after first of two daily transfections with control or a second Notch-1 siRNA. Immunoblots for EGFR (and α-tubulin loading control) after 48 h of exposure of 0206 cells to 25 μM of the gamma-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT) or equal volume:volume dimethyl sulfoxide (DMSO). (**C**) Immunoblots for EGFR (and GAPDH loading control) 72 h after first of two daily transfections with control or Notch-1 siRNA in osteosarcoma, colon cancer and human fibroblast cell lines. (**D**) Immunoblots for EGFR (and GAPDH loading control) 48 h after transfection with full-length Notch-1 construct or control plasmid pcDNA3.1 in U87MG cells.

AUGUUGUCCUGGAUG)d(TT) and control (from Xeragon/Qiagen)—r(UUCUCCGAACGUGUCACGU)d(TT), r(ACGUGACACGUUCGGAGAA)d(TT).

Reporter plasmid assays

A plasmid-encoding luciferase under the control of the EGFR promoter was kindly provided by Dr Alfred C.Johnson (Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD) (14). A p53 activity reporter construct encoding luciferase under the control of four consensus p53-binding sites was obtained from Panomics (Fremont, CA). Reporters of p53 promoter activity, with luciferase under the control of the p53 promoter, were kindly provided by Steven Safe (Texas A&M University, College Station, TX) (15) and Moshe Oren (Weizmann Institute of Science, Rehovot, Israel).

Cells were transfected with 1 μg of promoter–luciferase DNA or minimal TATA box-luciferase DNA and cotransfected with 50 ng beta-galactosidase plasmid (for normalization) 24 h after the second siRNA transfection (described above) using the Fugene 6 Transfection Reagent (Roche Diagnostics) as per the manufacturer's recommendations. Twenty-four hours after reporter plasmid transfection, cells were harvested and extracts prepared using luciferase cell lysis buffer (Promega Corp., Madison, WI). Luciferase activity was assessed with the luciferase assay system (Promega Corp.) as per the manufacturer's protocol on a $20/20^{n}$ Luminometer (Turner BioSystems, Sunnyvale, CA). Results of each experiment were normalized in two ways. Firstly, each well was divided by the beta-galactosidase activity for that well (determined by Galacto-Light kit, Tropix, Bedford, MA). Each experiment was performed with both the reporter plasmid of interest and a control luciferase plasmid with a minimal TATA box promoter; the luciferase–beta-galactosidase value for each reporter plasmid well was divided by the average luciferase–beta-galactosidase value of three control plasmid wells (the three which received the same siRNA or NICD/control plasmid). Reporter plasmid experiments were thus doubly normalized, with each sample corrected by its beta-galactosidase activity from a cotransfected constitutive vector and also by values in adjacent wells with a control reporter plasmid with luciferase driven by a minimal TATA box promoter.

Expression vectors

The pCLEN plasmid encoding the Notch-1 intracellular domain has been described previously and was a kind gift of G.Fishell (Department of Cell Biology, New York University School of Medicine, New York, NY) (16). The plasmid encoding wild-type p53 has been described previously and was a kind gift of B.Vogelstein (Molecular Genetics Laboratory, Johns Hopkins University Medical School, Baltimore, MD) (17). The full-length Notch-1 construct has also been described previously and was a kind gift of Spyros Artavanis-Tsakonas (Department of Cell Biology, Harvard Medical School, Boston, MA) (18). Cells were transfected twice with 2 μg of expression vector or empty pCRII-TOPO control (Invitrogen) 6 and 24 h after the second siRNA transfection (described above) using the Fugene 6 Transfection Reagent (Roche Diagnostics) as per the manufacturer's recommendations. For luciferase assays, cells were cotransfected with reporter constructs at the same time. Cells were harvested 24 h following transfection for luciferase assays or western blotting. For experiments with transfection of full-length Notch-1, U87MG cells were transfected with the Fugene HD reagent (Roche Diagnostics) as per the manufacturer's recommendations, using 2 μg of DNA and 4 μl of Fugene HD per well of a six-well plate.

Gamma-secretase inhibitor experiments

The 0206 primary human glioma cells were plated in laminin- and polyornithine-coated 6- or 12-well plates the day prior to adding drug. *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester, a well-established gamma-secretase inhibitor used frequently for Notch inhibition (19,20), was added the next day at 25 μM (from a stock solution in dimethyl sulfoxide of 10 mM). Control was equal volume:volume dimethyl sulfoxide.

Statistical analysis

Student's *t*-test was performed for most experiments with Microsoft Excel: mac2001 (Microsoft Corp., Redmond, WA). Deming regression was done (Figure 5) with Prism 4 (GraphPad Software, San Diego, CA). Deming regression was deemed a more appropriate method for this analysis than standard linear regression, since both values (Notch-1 and EGFR expression) have approximately equal errors of determination. All data shown are representative examples of results obtained from experiments conducted two or more times.

Results

Decreased EGFR expression with Notch-1 inhibition

Microarray data of glioma cell lines transfected with a previously validated Notch-1 siRNA (9) indicated downregulation of EGFR expression with Notch-1 knockdown. To better quantitate this, we determined EGFR mRNA and protein levels in two established glioma cell lines and the 0206 primary glioma cell line transiently transfected with control or Notch-1 siRNA. Both EGFR mRNA and protein dropped significantly with Notch-1 knockdown in all three cell lines (Figure 1A and B). Other glioma cell lines demonstrated similar downregulation of EGFR levels following Notch-1 inhibition (data not shown). To address concerns regarding the potential off-target effects of gene silencing by siRNA, we used a second siRNA targeted to a different site on Notch-1 and still observed a pronounced reduction in EGFR protein levels with Notch-1 knockdown (Figure 1B). We also demonstrated decreased EGFR protein in a glioma cell line treated with *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester, a small-molecule gamma-secretase inhibitor well established as a Notch inhibitor (Figure 1B). This experiment was performed in 0206, a primary glioma cell line derived and grown under serum-free conditions, as we have found that such cell lines are markedly more sensitive to gamma-secretase inhibitors than the standard glioma cell lines. The effect of Notch-1 knockdown was not restricted to gliomas, as we saw similar decreases in EGFR protein with Notch-1 silencing in osteosarcoma and colon cancer lines as well as in normal human fibroblasts (Figure 1C). Further corroborating the Notch-1 regulation of EGFR, transfection of U87MG glioma cells with Notch-1 upregulated EGFR protein expression (Figure 1D).

Effect of Notch-1 expression on EGFR promoter activity

We next considered whether Notch-1 was able to affect EGFR expression at the transcriptional level by regulating the activity of the EGFR promoter. Transfection with Notch-1 siRNA versus a control siRNA led to a 3- to 4-fold drop in activity of an EGFR promoter–luciferase reporter, suggesting transcriptional regulation of EGFR by Notch-1 (Figure 2A). We performed similar experiments with transient transfection of control vector or expression vector delivering NICD, the constitutively active Notch-1 intracellular domain, and observed an ∼3-fold induction of promoter activity following transfection (Figure 2B). This further indicated that Notch-1 affects EGFR transcription. These results were consistent in two glioma cell lines.

p53 is a mediator of the Notch-1 effects on EGFR expression

In seeking out potential mediators for the regulation of EGFR expression by Notch-1, we noted in our microarray data a decrease in p53 transcript levels following Notch-1 silencing. This was of interest because both wild-type and mutant p53 have been demonstrated to upregulate EGFR transcription (21–23). To confirm the effects of Notch-1 knockdown on p53 expression, we first assayed changes in p53 mRNA and protein levels by quantitative real-time PCR and western blotting, respectively (Figure 3A and B). Reductions in both p53 mRNA and protein expression were observed by these techniques. A reduction in p53 protein was also seen in 0206 primary glioma cells treated with the gamma-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (Figure 3B). Efficient transfection of U87MG cells with Notch-1 caused the reverse, an increase in p53 protein (Figure 3B). The decrease in p53 expression following Notch-1 knockdown led to a pronounced suppression in p53 transcriptional activity, comparable with that seen following transfection with a p53 siRNA, as assessed by a luciferase reporter plasmid under the regulatory control of the p53 consensus DNA-binding site (Figure 3C). Transfection with an NICD expression vector led to a corresponding induction of p53 activity (Figure 3C). As further evidence of Notch-1 regulation of p53 activity, we showed decreased expression of the well-established p53 targets p21, p53R2 and PCNA following Notch-1 silencing (Figure 3C).

Given our finding that p53 mRNA as well as protein was affected by Notch-1, we considered it likely that the regulation was at the transcriptional level. We therefore tested the effects of Notch-1 knockdown on the activity of p53 promoter–luciferase reporter plasmids. Transfection with Notch-1 siRNA indeed significantly decreased p53 promoter activity (Figure 3D). Upregulation of Notch-1

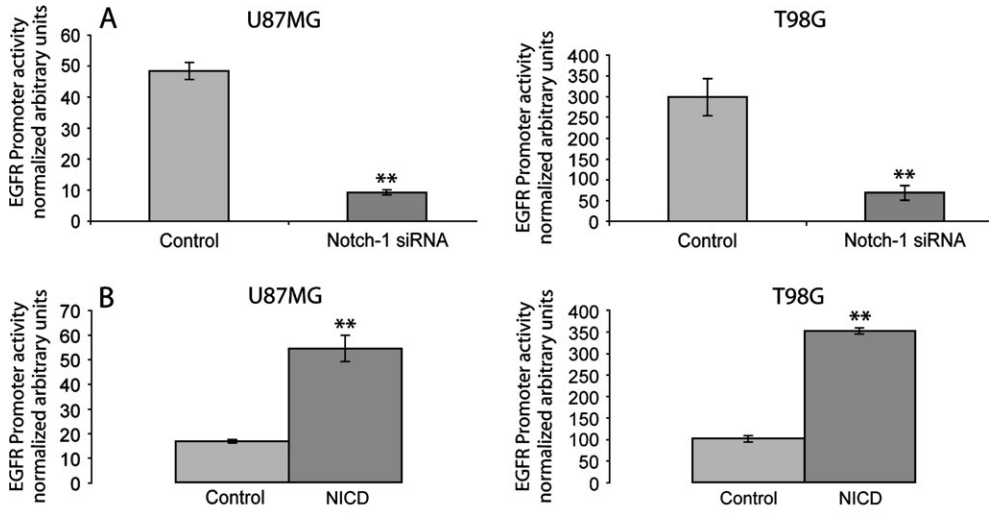

Fig. 2. Notch-1 regulation of EGFR promoter. (**A**) Double-normalized activity in two human glioma cell lines of EGFR promoter–luciferase reporter plasmid 72 h after the first of two daily transfections with control or Notch-1 siRNA. (**B**) Double-normalized activity in two human glioma cell lines of EGFR promoter–luciferase reporter plasmid 48 h after transfection with control or Notch-1 intracellular domain expression (NCID) plasmid. $^{**}P$ value <0.01.

activity through transfection of an NICD expression vector caused an increase in p53 promoter activity (Figure 3D).

In order to confirm that p53 regulates EGFR expression in glioma cells, we transfected U87 and T98 cells with a p53 siRNA that we validated by western blot (Figure 4A). As demonstrated in Figure 4A, p53 silencing resulted in decreased EGFR protein levels (Figure 4A). As has been demonstrated previously in other systems, p53 silencing by siRNA resulted in suppression of EGFR promoter activity (Figure 4B). Of note, these findings were seen in glioma cell lines that harbor either wild-type (U87MG) or mutant (T98G) p53 (24,25). In contrast, EGFR promoter activity was induced following the transient overexpression of wild-type p53 in U87MG cells (Figure 4B).

To further support a role for p53 in mediating the Notch-1 regulation of EGFR expression, we used a p53-null glioma cell line in addition to a well-established p53 knockout cell line. The effect of Notch-1 silencing on EGFR promoter–reporter activity was assessed in LNZ308, a glioma cell line lacking p53. No effect was seen in LNZ308 in comparison with the marked decrease noted in U87MG (Figure 4C). Furthermore, transient transfection with an NICD expression vector produced minimal change in EGFR reporter promoter activity in LNZ308 (Figure 4C), as compared with the previously noted increases in T98G and U87MG (Figure 2B). In contrast, transfection of LNZ308 by a p53 expression vector resulted in a marked increase in EGFR promoter activity (Figure 4C). In addition, we performed similar experiments in HCT116 colon carcinoma cells with wild-type p53 (HCT116 wt) or with both p53 alleles disrupted by homologous recombination (HCT116 $p53^{-/-}$) (generous gifts of B.Vogelstein). siRNA knock down of Notch-1 expression led to a significant decrease in EGFR promoter–reporter activity in HCT116 wt but not HCT116 $p53^{-/-}$ cells (Figure 4D). Transient transfection with the NICD expression vector yielded a much larger increase in EGFR promoter–reporter activity in HCT116 wt than in HCT116 $p53^{-/-}$ cells (Figure 4D). These results indicate the importance of p53 for Notch-1 responsiveness of the EGFR promoter.

Correlation of Notch-1 and EGFR expression in primary human high-grade astrocytomas

We ascertained the relevance of our findings concerning the relationship between Notch-1 and EGFR expression to primary human gliomas by assessing Notch-1 and EGFR transcript levels using quantitative PCR in a panel of 22 primary human high-grade astrocytomas described previously (9). To eliminate the potential confounder caused by the relatively frequent amplification of EGFR in glioblastomas, we excluded samples with more than three copies of EGFR as determined by single nucleotide polymorphism array via methods described previously (26). As was suggested by our *in vitro* data, there was a significant positive correlation between EGFR and Notch-1 mRNA levels in these tumors (Figure 5, P value <0.05).

Discussion

In this report, we demonstrate the transcriptional regulation of EGFR by Notch-1. In addition to demonstrating the effects of Notch-1 expression on EGFR mRNA and protein levels in various glioma cell lines, we also demonstrate a similar correlation between EGFR mRNA and Notch-1 mRNA in patient-derived high-grade glioma samples. Additionally, a similar relationship was demonstrated in human fibroblasts and in other human cancer cell lines. As there are no classical CBF1 target sites in the EGFR promoter indicating more direct regulation by Notch-1, we investigated potential mediators of this regulation. The p53 protein has been well established as a transcriptional activator of EGFR expression, and interestingly this has been demonstrated for both wild-type and mutant versions of p53. Mutant p53 may retain transcriptional activity through binding to Sp1 protein (27), and Sp1 is a critical regulator of EGFR transcription (28,29). Our data confirmed upregulation of EGFR by p53 in glioma cell lines with both wild-type (U87MG) and mutant (T98G) p53. We noted potent regulation of p53 expression and activity by Notch-1, identifying it as a potential mediator of the Notch-1 regulation of EGFR.

There are no earlier reports documenting a role for Notch in upregulating EGFR expression. It has previously been found that Notch activation is key in maintaining Ras pathway activity, one of the downstream mediators of EGFR (30). Our report suggests that Notch also regulates the Ras pathway farther upstream through regulation of EGFR expression. Notch and EGFR have been found to have complementary expression patterns in *Drosophila* development (31,32), differing from our findings in human cells. While not showing regulation of one by the other, a previous report showed a correlation of Notch-3 and EGFR levels in human lung cancer and also suggested Notch-3–EGFR pathway interaction (33). Previous links between Notch and p53 have described variable interactions. Two reports mention an increase in p53 expression with constitutive Notch activation (34,35), but suppression of p53 by Notch activity has also been well established (36,37). No previous report to our knowledge has demonstrated a major decrease in p53 expression and activity with Notch

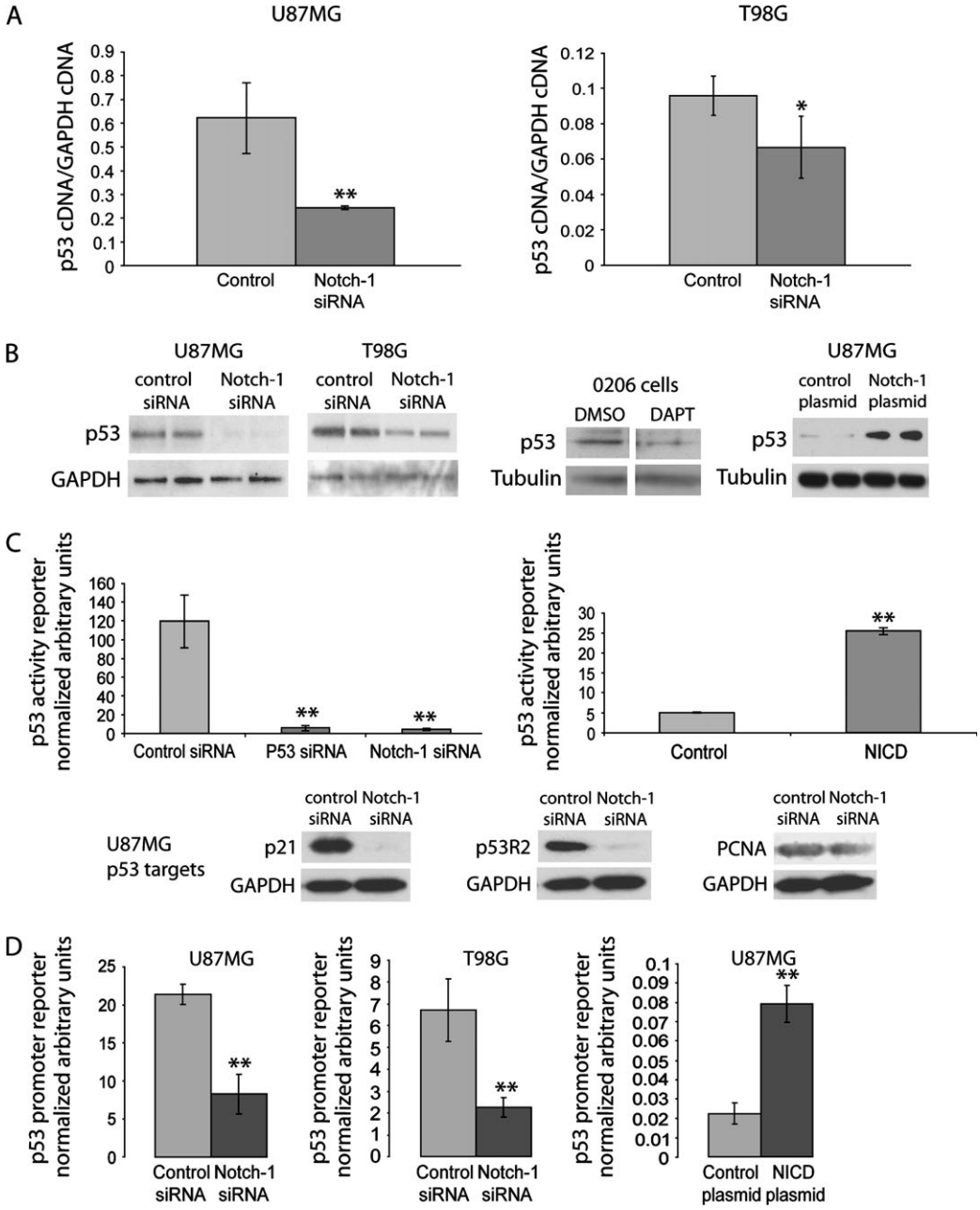

Fig. 3. Notch-1 regulates p53 expression and transcriptional activity. (**A**) Real-time PCR of p53 mRNA from two glioma cell lines 72 h after first of two daily transfections with control or Notch-1 siRNA. $^{*}P$ value <0.05 and $^{**}P$ value <0.01. (**B**) Immunoblots for p53 (and GAPDH loading control) from two human glioma cell lines 72 h after first of two daily transfections with control or Notch-1 siRNA. Immunoblots for p53 (and α-tubulin loading control) after 48 h of exposure of 0206 cells to 25 μM of the gamma-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT) or equal volume:volume dimethyl sulfoxide (DMSO). Immunoblots for p53 (and GAPDH loading control) 48 h after transfection with full-length Notch-1 construct or control plasmid pcDNA3.1. Immunoblots for p53 (and GAPDH loading control) 48 h after transfection with full-length Notch-1 construct or control plasmid pcDNA3.1. (**C**) Double-normalized activity in two human glioma cell lines of a p53 activity reporter plasmid 72 h after the first of two daily transfections with control, p53 or Notch-1 siRNA. Double-normalized activity in two human glioma cell lines of a p53 activity reporter plasmid 48 h after transfection with control or NICD expression plasmid. Immunoblots for p21, p53R2 and PCNA (and GAPDH loading controls) 72 h after transfection with control or Notch-1 siRNA. $^{**}P$ value <0.01. (**D**) Double-normalized activity in two human glioma cell lines of a p53 promoter–luciferase reporter plasmid 72 h after the first of two daily transfections with control or Notch-1 siRNA. Double-normalized activity in U87MG of p53 promoter–luciferase reporter plasmid 48 h after transfection with control or NICD expression plasmid. $^{**}P$ value <0.01.

inhibition, as in this report. The difference between our findings and those of prior reports claiming suppression of p53 by Notch activity may be secondary to context or dose dependence of Notch activity.

Context dependence of Notch pathway activity is widely accepted. While we found Notch-1 knockdown to result in decreased EGFR in gliomas and other cell lines we assessed, it may not be true in all settings. Some recent reports indicate that Notch signaling may have very different outputs depending on whether Delta-like or Jagged ligands are binding (38–41), and this difference may be one explanation for context dependence. While it is not described in this report, we assessed the effects of Delta-like-1 and Jagged-1 knockdown on EGFR protein levels in glioma cell lines. Jagged-1 knockdown, like Notch-1 knockdown, markedly decreased EGFR protein, whereas

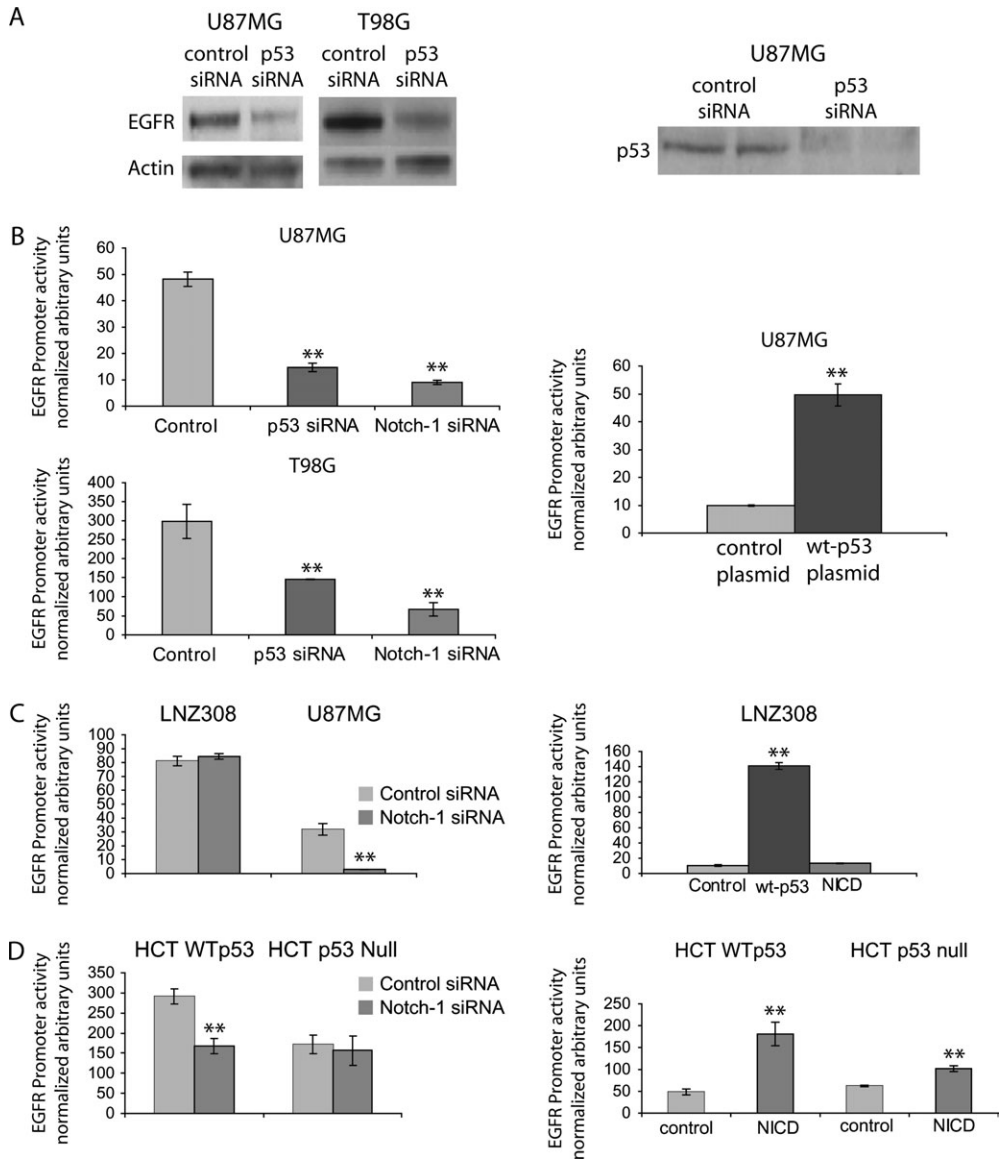

Fig. 4. p53 upregulates EGFR transcription in glioma cells and is a mediator of Notch-1 regulation of EGFR expression. (**A**) Immunoblots for EGFR (and actin loading control) from two human glioma cell lines 72 h after first of two daily transfections with control or p53 siRNA. Immunoblot for p53 from U87MG glioma cells 72 h after transfection with control or p53 siRNA. (**B**) Double-normalized activity in two human glioma cell lines of EGFR promoter–luciferase reporter plasmid 72 h after the first of two daily transfections with control, p53 or Notch-1 siRNA. Double-normalized activity in U87MG of EGFR promoter–luciferase reporter plasmid 48 h after transfection with control or wild-type p53 expression plasmid. (**C**) Decreased double-normalized EGFR promoter–reporter activity in a p53 wild-type glioma cell line (U87MG) but not in a p53-null glioma cell line (LNZ308) 72 h after the first of two daily transfections with control or Notch-1 siRNA. EGFR promoter–reporter activity in a p53-null glioma cell line 48 h after transfection with control, p53 or NICD expression vector. (**D**) EGFR promoter–reporter activity in p53 wild-type and null variants of a colon cancer cell line 72 h after first of two daily transfections with control or Notch-1 siRNA. EGFR promoter–reporter activity in p53 wild-type and null variants of a colon cancer cell line 48 h after transfection with control or NICD expression plasmid. $^{**}P$ value <0.01.

Delta-like-1 knockdown slightly increased it (data not shown). It is therefore possible that it is Jagged-1–Notch-1 signaling and not Delta-like-1–Notch-1 signaling which maintains EGFR expression, although this hypothesis awaits formal testing.

Cell lines lacking p53 show a diminished but still present level of regulation of EGFR by Notch-1. Thus, while we clearly identify p53 as a mediator of the Notch-1 regulation of the EGFR promoter, there are probably other mediators, possibly posttranscriptional, yet to be identified.

It should be noted that the transcriptional upregulation of EGFR by Notch activity is probably less potent than that from EGFR amplification, a characteristic of roughly 40% of glioblastomas (42). EGFR amplification may increase its expression by 10- to 40-fold, potentially overwhelming any effects from Notch regulation. We therefore eliminated as potential confounders any samples with EGFR amplification from our analysis of Notch–EGFR expression correlation in primary human samples in Figure 5. However, given that the EGFR amplicon still contains the normal EGFR promoter, we hypothesize that Notch regulation still affects EGFR expression levels in tumors with amplification.

This work has significant implications for both oncology and developmental research. The Notch pathway is ubiquitous in development and cell fate determination, and both EGFR and p53 play roles in development as well. This report suggests EGFR and p53 as mediators of some of the many roles of Notch in development. It also establishes another point of cross talk between the Notch and EGFR–Ras pathways. Notch is currently being investigated as a therapeutic target in several cancers, including glioma, and this work

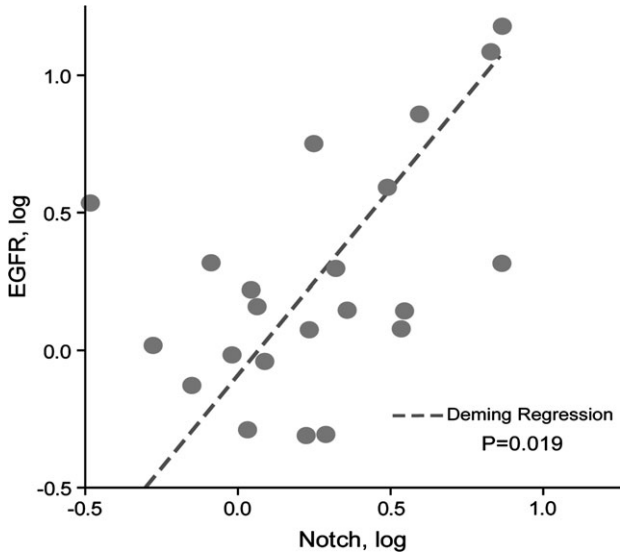

Fig. 5. Levels of Notch-1 and EGFR mRNA correlate in primary human glioma samples. Real-time PCR with Notch-1 and EGFR probes of mRNA from primary human high-grade gliomas is shown. Results are normalized relative to amount of 18S rRNA and the results are plotted by log of the amount relative to reference sample. Samples with EGFR amplification are excluded. Deming regression was performed to plot line and indicates significance.

suggests diminished EGFR expression as an additional mechanism of action. Optimism for Notch inhibition may need to be tempered, however, by the theoretical concern raised by the potential implications of long-term p53 downregulation that might result in some contexts from chronic Notch inhibition.

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Conflict of Interest Statement: None declared.

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